Fungal Endophyte Removal Does Not Reduce Cold Tolerance of Tall Fescue

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Abbreviations: E−, endophyte-free; E+, endophyte-infected.

ABSTRACT

Tall fescue [*Schenodorus phoenix* (Scop.) Holub.] has historically been of minor importance to livestock agriculture in cooler regions of the temperate zone. As its use in these regions expands, it becomes increasingly important to understand the effect of infection by its fungal endophyte (*Neotyphodium coenophialum* Morgan-Jones and Gams) on agronomic fitness of tall fescue. Our objective was to determine the effect of endophyte infection status on forage yield and survival of tall fescue under field conditions in which freezing temperatures, combined with lack of persistent snow cover, constitute the most significant stress factor. In Experiment 1, we evaluated 427 half-sib families of four tall fescue cultivars, half endophyte-infected (E+) and half endophyte-free (E−), for forage yield and survival. There was no effect of endophyte removal on either forage yield or survival, despite complete stand loss of one cultivar during two winters. In Experiment 2, we evaluated 640 clones of confirmed endophyte status, half E+ and half E−, for survival under two management regimes at four Wisconsin locations. Results were nearly identical to those for Experiment 1, with complete mortality in the unadapted cultivar and high survival rates in the three adapted cultivars, regardless of endophyte status, management, or location. In conclusion, we found no evidence that endophyte removal resulted in any disadvantage to tall fescue host-plant survival under harsh winter conditions. In regions where freezing temperatures and desiccating winds provide the only or most important abiotic stress factor, removal of endophytic fungi do not appear to reduce fitness of their tall fescue host.

Tall fescue [*Schenodorus phoenix* (Scop.) Holub.] has historically been of minor importance to livestock agriculture in cooler regions of the temperate zone. However, with increased interest in grazing systems in the northern United States and Canada, there is heightened interest in the use of grasses that can withstand grazing pressures and maintain acceptable production levels through the warmer and drier portions of the growing season. Tall fescue is increasingly seen as one of the solutions to this problem.

Tall fescue is host to an endophytic fungus (*Neotyphodium coenophialum* Morgan-Jones and Gams) that aids the host's ability to survive under environmental stresses (Bacon and Siegel, 1988). Endophyte-infected (E+) plants have enhanced seedling performance and survival, increased drought tolerance, increased nitrogen assimilation, decreased insect herbivory, and decreased nematode damage compared to endophyte-free (E−) plants (Pedersen et al., 1990; Joost, 1995). The presence of endophyte in the host increases root biomass (De Battista et al., 1990) and alters root morphology (Malinowski et al., 1999) of tall fescue. These responses appear to contribute to enhanced drought avoidance (Hill et al., 1996; White et al., 1992) and nutrient assimilation (Cheplick et al., 1989; Arachavaleta et al., 1989) of E+ tall fescue plants. Drought tolerance also appears to be enhanced by higher carbohydrate accumulation in E+ plants (Richardson et al., 1992) and an enhanced osmoregulatory

system capable of maintaining higher turgor pressure under drought (Bacon, 1993; Elbersen and West, 1996; Elmi and West, 1995; Richardson et al., 1993).

Because E+ is the natural state for a tall fescue host, endophyte effects in E+ vs. E− comparative studies (e.g., Arachavaleta et al., 1989; Elmi and West, 1995) should properly be labeled as "endophyte removal effects." More recent studies, using so-called "friendly" endophytes in which E− plants are reinfected with the fungal endophyte (e.g., Bouton and Easton, 2005; Hopkins and Alison, 2006) refer to "endophyte infection effects." The effect of endophyte presence on forage yield and survival of tall fescue is very much a function of environmental conditions. Under drought and/or heat-stress conditions, both survival and forage yield are higher in E+ than in E− plants (Arachavaleta et al., 1989; Bouton et al., 1993a; Asay et al., 2001), possibly due to enhanced tiller survival under drought (West et al., 1993). However, under relatively stress-free conditions, both survival and forage yield of tall fescue was unaffected by endophyte status (Asay et al., 2001). Host responses to endophyte presence are dependent on the genotypic background of both the host (Belesky et al., 1989; De Battista et al., 1990) and the endophyte (Hill et al., 1996).

Because the fungal endophyte causes drastic reductions in livestock health and performance (Stuedemann and Hoveland, 1988), the tall fescue seed industry has shifted toward E− cultivars or cultivars infected with endophyte genotypes benign to livestock. Endophyte-free cultivars present unique problems to plant breeders who may be faced with the task of initiating breeding programs with germplasm that is largely unadapted to stressful environments (Pedersen and Sleper, 1988; Bouton et al., 2001).

As tall fescue moves into the cooler regions of temperate North America a key question remains unanswered: does the removal of the endophyte from its tall fescue host reduce survival, forage yield, or other fitness traits in regions that are characterized by relatively stress-free summers, but severe cold and freezing stress during winter? Although the endophytic fungus (*Neotyphodium lolii* Latch, Christensen, and Samuels) of perennial ryegrass (*Lolium perenne* L.) also confers drought tolerance, heat tolerance, and insect-herbivory resistance to its host, it has relatively little effect on host survival and fitness in relatively stress-free cool-season environments (Eerens et al., 1992, 1997, 1998a, 1998b). Our objective was to determine the effect of endophyte removal on forage yield and survival of tall fescue under field conditions in which freezing temperatures, combined with lack of persistent snow cover, represent the most significant stress factor.

MATERIALS AND METHODS
Germplasm
The experimental population AU Vigor is a 15-clone synthetic (van Santen, 1992), of which eight clones trace back to southern European or North African germplasm introduced to NPGS-GRIN via Australia, one clone traces back to KY-31, and six are of unknown provenance but likely have a Mediterranean background. Both AU Vigor E+ and E− are characterized by active growth during winters in the southeastern United States (van Santen, personal observation). Cultivars GA-5 and Jesup were obtained as both E+ and E− versions from Dr. Joe Bouton, formerly at the University of Georgia, Athens. Both cultivars can be traced to long-term KY-31 pastures in the Southeast (GA-5) or a particular pasture in Georgia (Jesup) (Bouton et al., 1993b, 1997). The breeding program of International Seeds, Inc. (Halsey, OR; now DLF International Seeds, Halsey, OR) provided KY-31 E+ and KY-31 E−. More than 60 yr after its release (Fergus and Buckner, 1972), most pastures in the eastern United States still contain a high proportion of KY-31 E+ (Easton and Fletcher, 2007; Rudgers and Clay, 2007) in spite of the detrimental effect of the fungal endophyte on the performance of grazing animals.

Isolated clonal spaced-plant nurseries were established near Brownsville, OR, in autumn 1991 by planting E+ and E− versions of a given population in alternate rows. Rows were color-coded and plants harvested and processed separately. Equal amounts of seed from all plants within a population × endophyte status combination were combined to create the eight bulk subpopulations. The term isofrequent populations has been applied to these subpopulations to indicate that gene frequencies between the two subpopulations are equal (van Santen, 1994). We determined endophyte infection percentages of bulked subpopulations (Table 1) by histochemical staining (Clark et al., 1983) of 100 seedlings per subpopulation.

Experiment 1
A total of 880 plots were planted in April 1996 at Arlington, WI (Plano silt loam [fine-silty, mixed, mesic Typic Argiudoll]). The experimental design was a randomized complete block with two replicates and a split-plot randomization restriction in which base populations were whole plots and half-sib families were subplots. The number of half-sib families per population ranged from 38 to 58 (Table 2). Endophyte-free and E+ families were randomized together within each whole plot. Up to 16 plots of each check population (AU Triumph, Cajun, and the eight bulk populations) were planted to make up a total of 110 plots within each whole plot. Plots were seeded at a rate of 13 kg ha^{-1} in five drilled rows that were 15 cm apart with a total dimension of 0.9 by 1.2 m. All plots established well and persisted throughout the first growing season in which they were clipped three times to control annual weeds.

In 1997 and 1998, plots were fertilized with 90 kg N ha^{-1} in early spring and after each of the first two harvests. Plots were harvested at a 9-cm cutting height with a flail-type harvester in early June, early August, and late October of 1997 and 1998. A 500-g sample of forage was sampled for dry matter determination and forage yields were adjusted to a dry-matter basis. Plots were cut without data collection in early June 1999. Ground cover was measured immediately after first harvest in 1997 through 1999 using a 50-cell grid in which each cell had a dimension of 9 by 12 cm and the grid covered the entire plot. Ground cover was scored from 0 to 50, as tall fescue present or absent within each grid cell, then converted to a percentage as later described by Vogel and Masters (2001).

In 1997, plots that had suffered from significant winter injury were largely taken over by annual weeds, which made up the bulk of the forage yield measurements for those plots. Therefore, before the initiation of new growth in April 1998 and 1999, all plots were sprayed with 4.5 kg a.i. ha^{-1} pendimethalin [N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine]. Annual weeds that appeared later in the 1998 growing season were removed by hand or by application of 2.2 kg a.i. ha^{-1} 2,4-dichlorophenoxyacetic acid, restricting all measured forage yield to tall fescue planted in each plot.

Forage yield and ground cover data were analyzed by analysis of variance in which replicates and families were random effects and populations and endophyte status were fixed effects. Population means were tested by Fisher's least significant difference.

Experiment 2

In 2003, we used a commercial immunoblot kit (Hiatt et al., 1999) sold by Agrinostics Ltd. Co. (Watkinsville, GA) to screen seedlings from the eight populations for the presence of the endophyte. All plants with verified endophyte status were mechanically split into 10 clonal ramets (replicates) of 1 to 2 tillers each. Within each of the eight base population–endophyte status combinations, 80 genotypes (640 total genotypes) were chosen at random from those that were successfully split into 10 vigorous clonal ramets. At the time of transplanting in May 2005, each transplant had approximately six to eight tillers.

In August 2004, soil at four locations was cultivated and planted to a fine fescue turf mixture made up of approximately equal parts *Festuca rubra* ssp. *rubra* L., *F. rubra* ssp. *commutata* Gaudin, and *F. trachyphylla* (Hackel) Krajina seeded at a rate of 182 kg ha^{-1}. The four locations, all in Wisconsin, were Arlington (Plano silt loam), Lancaster (Fayette silt loam, fine-silty, mixed, mesic Typic Hapludalf), Prairie du Sac (Richwood silt loam; fine-silty, mixed, mesic Typic Argiudoll), and Marshfield (Withee silt loam; fine-loamy, mixed, superactive, frigid Aquic Glossudalf). In April 2005, experimental areas were arranged on the fine fescue turf at each location and the future site of each tall fescue transplant was marked with a small spot of spray paint. Glyphosate was used to kill a 15-cm circle of fine fescue turf centered on each spot of spray paint.

After the treated fine fescue turf circles were clearly dead, tall fescue plants were transplanted into the four fields in May 2005. Two experiments were planted at each location, one to be managed as a turf and one to be managed as a forage crop. Each of the eight location–management combinations received one clonal ramet of each genotype so that all 640 genotypes were represented by two clonal ramets at each location (one for turf management and one for forage management). Within each location–management combination, the experimental design was a randomized complete block with 10 replicates and a split-split-plot randomization restriction, with base populations as whole plots, endophyte status as subplots, and genotypes as sub-subplots. Each replicate consisted of 64 genotypes (eight within each base population–endophyte status combination), which were randomly assigned to each of the 10 replicates. Each sub-subplot was a single plant, spaced

30-cm from adjacent plants, and each subplot was a row of eight plants, spaced 0.9 m from adjacent rows.

Plants were watered immediately after transplanting and carefully monitored for the first 6 wk following transplanting. Plants that died during this 6-wk period were replaced with another clonal ramet of the same genotype from reserve stock in the greenhouse. Bare soil was kept weed-free by occasional hand weeding. During 2005 and 2006, plots were managed according to a turf or a forage-crop management. Turf management, initiated approximately 6 wk after transplanting, consisted of frequent mowing at a height of 8 cm, generally not allowing tall fescue plants to reach a height greater than 15 cm above soil level. Forage management consisted of two harvests in 2005 (mid-August and late October) and three harvests in 2006 (early June = early heading growth stage, early August, and late October) with a flail-type harvester at a height of 9 cm. Each of the 5120 plants was scored for survival (0 = dead, 1 = alive) in September 2005 and 2006 and May 2006 and 2007.

Survival percentages were computed for each subplot of eight plants and were analyzed by analysis of variance in which replicates and families were random effects and populations and endophyte status were fixed effects. Population means were tested by Fisher's least significant difference.

Daily minimum temperatures for the period 1 November through 30 April for the 11 location-years in the two experiments were obtained from the Wisconsin State Climatology

Table 1. Characterization of tall fescue seed produced near Brownsville, OR, in 1992.

Population	Endophyte status[†]	100-seed mass	Seed germination	Infected seedlings
		mg	——— % ———	
AU Vigor	E–	310	91	8
AU Vigor	E+	280	99	94
GA 5	E–	320	98	8
GA 5	E+	300	96	56
Jesup	E–	310	92	0
Jesup	E+	320	99	94
KY-31	E–	350	96	0
KY-31	E+	280	97	76

[†]E–, endophyte-free; E+, endophyte-infected.

Table 2. Means over all half-sib families within endophyte-free (E–) and endophyte-infected (E+) isofrequent populations of tall fescue evaluated for forage yield and ground cover at Arlington, WI.

Population	Endophyte status	No. of families	Forage yield		Ground cover		
			1997	1998	1997	1998	1999
			— Mg ha^{-1} —		——— % ———		
AU Vigor	E+	41	5.00	0.00	2	0	0
AU Vigor	E+	58	5.50	0.00	5	0	0
GA 5	E–	38	13.45	13.24	100	100	100
GA 5	E+	58	13.59	13.07	100	100	100
Jesup	E–	58	14.35	13.23	100	100	100
Jesup	E+	58	14.73	13.93	99	99	99
KY-31	E–	58	14.06	13.33	100	100	99
KY-31	E+	58	14.38	13.69	100	100	100
LSD$_{0.05}$			0.69	0.65	1	1	1

Office (http://www.aos.wisc.edu/~sco/) (Fig. 1 and 2). Daily snow cover data for the same time periods were obtained from the U.S. Department of Commerce National Climate Data Center (http://www.ncdc.noaa.gov/oa/ncdc.html) (Fig. 3).

RESULTS AND DISCUSSION
Experiment 1

Endophyte status had little or no effect on either forage yield or ground cover of all four tall fescue populations, measured as half-sib families (Table 3). AU Vigor experienced nearly complete stand loss during the first winter after planting, regardless of its endophyte status. The second winter removed any surviving plants so that there were no tall fescue plants or vegetative growth on the herbicide-treated and hand-weeded plots in 1998. Ground cover of the remaining three tall fescue cultivars was unaffected by severe winter conditions, which included frequent severe freezing temperatures and sporadic snow

cover (Fig. 1 and 3). There was some genetic variability for forage yield among these three populations, and the lack of biologically significant winter injury indicated that this variability reflected differences in yield potential, not differences in survival potential. In general, genetic variability among half-sib families for forage yield or ground cover was not significant within these four cultivars (data not shown).

Results for the eight bulk populations of half-sib families (four cultivars with E+ or E−) were very similar (Table 3). The only exception was a significant reduction in forage yield for KY-31 E− compared to KY-31 E+ in 1998. Apart from this observation, the bulk populations provided the same conclusion as the half-sib families: endophyte status had little or no effect on persistence or forage yield potential of tall fescue in a cold-temperate environment. Because the bulk populations had forage yields similar to that of the check cultivars, both of which have performed well in forage-yield trials in this region (Casler et al., 1998), GA 5, Jesup, and KY-31 all appear to be well adapted to this region.

Three characteristics of this experiment prevent any definitive conclusions about the effect of endophyte infection on cold tolerance of tall fescue. First, the use of only one location limits inferences for this experiment. Second, the lack of confirmation of endophyte infection on plants within the E+ half-sib family plots leaves some level of doubt about biological interpretation of the E+ vs. E− comparisons. Third, because ground cover was rated only once per year, we could not conclusively determine that all or most plant mortality occurred during the winter months. Nevertheless, the results of this experiment led directly to the design and implementation of Experiment 2.

Experiment 2

Following establishment of 5120 tall fescue plants under irrigation in May 2005, plant mortality numbered 82 plants in summer 2005, 1276 plants in winter 2005–2006, 11 plants in summer 2006, and 176 plants in winter 2006–2007, for a total of 1545 plants (30.2% mortality averaged across locations and cultivars). A total of 6% of all mortality occurred during the growing season and 94% occurred during winter, indicating that cold or freezing stress is the most likely reason for
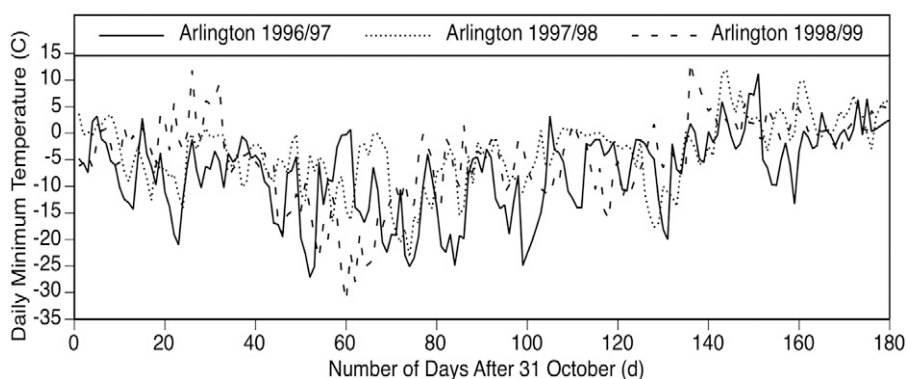

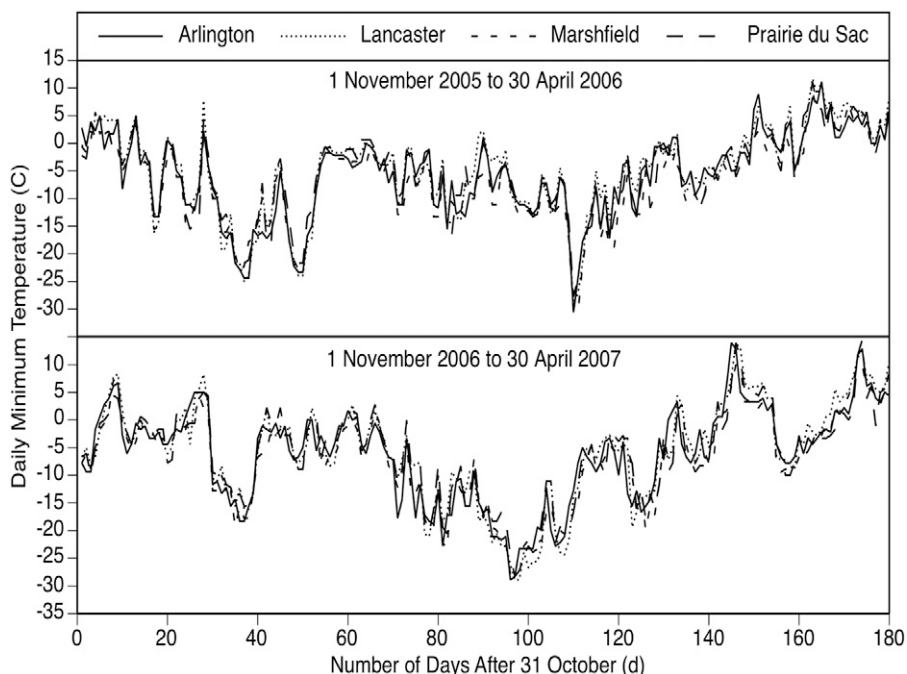Figure 1. Daily minimum air temperatures from 1 November through 30 April at Arlington, WI, for the duration of Experiment 1.

Figure 2. Daily minimum air temperatures from 1 November through 30 April at four Wisconsin locations for the duration of Experiment 2.

most tall fescue mortality in this experiment. Most of the plants of AU Vigor died during the first winter and the remainder died during the second winter, mimicking the results observed in Experiment 1 (Fig. 4). For the other three cultivars, there was a small amount of mortality during both winters, but all three cultivars had at least 89% survival two years after transplanting.

Following the first winter (May 2006 scoring date), and for the last two scoring dates (October 2006 and May 2007), the only significant effects in the analysis of variance were location, cultivar, and location × cultivar. These effects together accounted for 99.7, 99.8, and 99.9% of the total sums of squares for the ANOVAs of the last three scoring dates, respectively. Cultivar was the largest of these effects, accounting for 99.1 to 99.2% of the sums of squares, due to the severe mortality within AU Vigor. By May 2007, all plants of AU Vigor had died, regardless of location (Table 4). The small location × cultivar interaction was due to a greater instability of Jesup compared to GA 5 and KY-31, resulting in greater variability among locations for this cultivar compared to the other two cultivars. This may reflect the narrower genetic base for Jesup, whose 15 parental clones all came from a single KY-31 pasture (Bouton et al., 1997), whereas the five parental clones of GA 5 were originated from a much broader geographic area (Bouton et al., 1993b). Management and interactions of management with other factors in the ANOVAs had no effect on survival of tall fescue in this study. There were no effects of endophyte status, nor were there any interactions of endophyte status with any of the other factors in the ANOVAs. As observed in Experiment 1, endophyte status had no impact on survival of tall fescue, regardless of whether the cultivar was adapted or unadapted to these locations, which were all characterized by frequent severe freezing temperatures and sporadic snow cover (Fig. 2 and 3).

## SYNTHESIS AND CONCLUSIONS

The results of this study confirm that some cultivars of tall fescue are adapted to cold climates in which snow

Table 3. Means of endophyte-free (E–) or endophyte-infected (E+) isofrequent bulk populations and two check cultivars of tall fescue evaluated for forage yield and ground cover at Arlington, WI.

Population	Endophyte status	Forage yield		Ground cover		
		1997	1998	1997	1998	1999
		— Mg ha^{-1} —		— — — — % — — — —		
AU Vigor	E–	4.93	0.00	3	0	0
AU Vigor	E+	4.77	0.00	5	0	0
GA 5	E–	14.84	14.26	100	100	100
GA 5	E+	14.68	14.17	100	100	100
Jesup	E–	15.44	14.51	100	100	100
Jesup	E+	15.61	14.71	100	100	100
KY-31	E–	14.76	13.83	100	100	100
KY-31	E+	14.97	14.96	100	100	99
AU Triumph	E–	14.25	14.29	100	100	100
Cajun	E–	14.66	14.46	100	100	100
LSD$_{0.05}$		1.77	1.02	2	<1	1
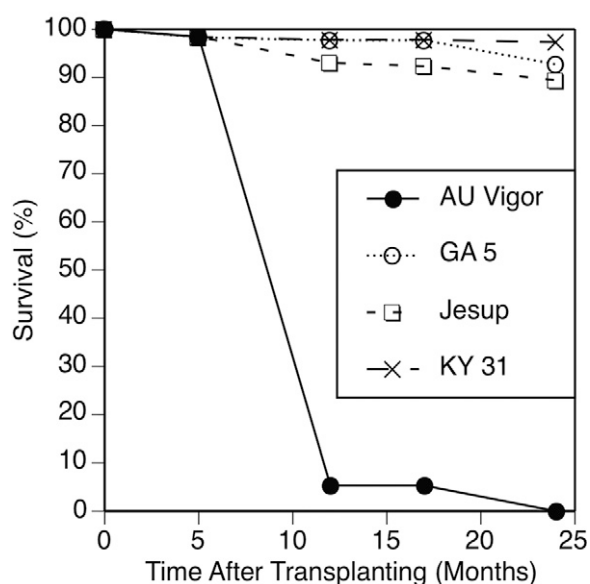
Figure 4. Survival percentages of four tall fescue cultivars evaluated at four Wisconsin locations (averaged over locations) following establishment of transplants in May 2005 (LSD$_{0.05}$ = 1.1, 1.5, 1.5, and 1.6% at 5, 12, 17, and 24 mo after transplanting).
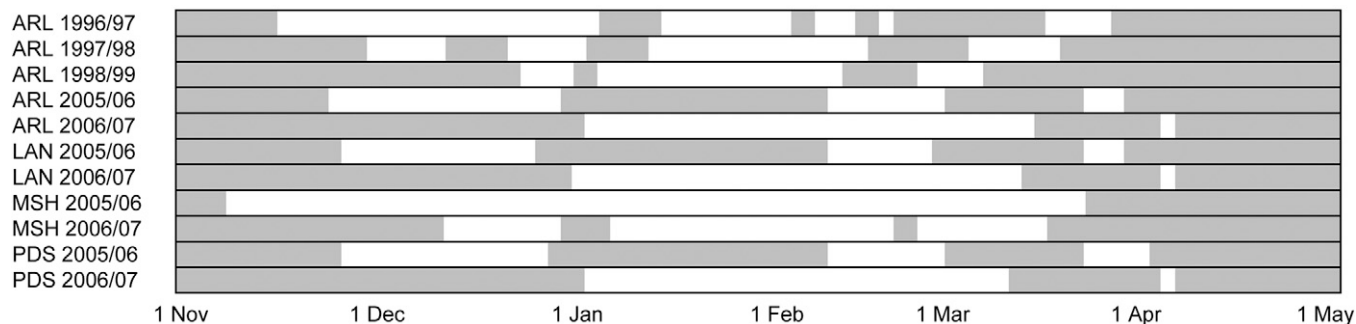
Figure 3. Daily snow cover (minimum 2 cm) from 1 November through 30 April for 11 location-years pertaining to both Experiment 1 (Arlington 1996–1999) and Experiment 2 (four locations in 2005–2007). Gray areas indicate open ground and white areas indicate ≥2 cm of snow cover on a daily basis (ARL, Arlington; LAN, Lancaster; MSH, Marshfield; PDS, Prairie du Sac).

Table 4. Mean survival of endophyte-free (E–) or endophyte-infected (E+) isofrequent tall fescue populations evaluated 24 mo after transplanting at four Wisconsin locations in May 2007.

Population	Endophyte status	Arlington	Lancaster	Marshfield	Prairie du Sac	Mean
		————————— % —————————				
AU Vigor	E–	0.0	0.0	0.0	0.0	0.0
AU Vigor	E+	0.0	0.0	0.0	0.0	0.0
GA 5	E–	90.6	94.4	93.1	90.0	92.0
GA 5	E+	90.6	95.0	95.0	92.5	93.3
Jesup	E–	87.5	98.8	91.9	83.1	90.3
Jesup	E+	85.0	98.1	91.3	79.4	88.4
KY-31	E–	97.5	98.1	95.6	97.5	97.2
KY-31	E+	98.8	98.1	96.9	95.6	97.3
LSD$_{0.05}$		3.0	4.2	4.1	4.6	2.0

for four tall fescue cultivars originating from either Mediterranean or continental European germplasm tested under harsh winter conditions. In regions where freezing temperatures and desiccating winds provide the most important abiotic stress factor, endophytic fungi do not offer a fitness advantage to tall fescue host cultivars that are sufficiently adapted to survive these conditions nor to those that are highly unadapted to these conditions.

Acknowledgments

cover is absent or sporadic. A previous study also demonstrated high survival rates of numerous tall fescue cultivars evaluated at multiple locations with persistent freezing temperatures and minimal snow cover (Casler et al., 1998). In that study, tall fescue cultivars had survival rates nearly equal to cultivars of meadow fescue, *L. pratense* (Huds.) Darbysh., its close relative that originates from higher latitudes and higher elevations in Europe and Asia. The lack of cold or freezing tolerance of AU Vigor was likely due to the use of primarily Mediterranean germplasm in development of this cultivar (see Materials and Methods).

There is overwhelming evidence that the presence of the endophytic fungus in tall fescue protects its host from numerous biotic and abiotic stresses, including drought stress, light stress, mineral stress, and insect herbivory (Malinowski et al., 2005; Popay and Bonos, 2005). In contrast, we found no evidence to support an effect of endophyte removal on cold or freezing tolerance of tall fescue in a region that is typically characterized by persistent freezing temperatures, harsh and persistent (desiccating) winds, and minimal or sporadic snow cover (Fig. 3). In this regard, tall fescue seems to behave in a manner similar to that of perennial ryegrass. In a cool-moist environment, endophyte status had no effect on numerous growth and production characteristics of perennial ryegrass, except under conditions of extreme drought (Eerens et al., 1992, 1997, 1998a, 1998b). Studies on the effect of endophyte status on freezing tolerance of meadow fescue have not been reported. Susceptibility to snow mold fungi (*Typhula* spp.) is one major component of winter survival in the fescues and ryegrasses, but the role of snow mold in regulating winter survival status of E+ vs. E– meadow fescue is unclear due to inconsistent results related to growth environments, host genetic background, and pathogen genotype (Wäli et al., 2006).

In conclusion, we found no evidence that endophyte removal confers any disadvantage to host-plant survival

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